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(54) **Method of purifying ologonucleotide from biological samples**

(57) Methods are provided for purifying oligonucleotides from biological samples using magnetically responsive particles. The method involves incubating magnetically responsive particles, to which oligonucleotide-binding partners have been linked, with biological sample preparations to form a particle-binding partner-analyte complex. The complex is then separated from the cellular debris by application of a magnetic field.

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molecular weight DNA compared with DNA isolated using conventional techniques. The processing takes at least a day.

Majumdar et al. (1991) *BioTechniques* 11:940-101 describes a method of isolating DNA by lysing cells with a detergent, extracting the oligonucleotide from the aqueous sample with phenol:chloroform:isoamyl alcohol and eluting the DNA from the organic phase by increasing the pH.

SUMMARY OF THE INVENTION

To address the above-mentioned need in the art, the invention disclosed and claimed herein provides a method for preparing highly purified oligonucleotides which are adequate for clinical assays and other assays which require oligonucleotides purified to remove contaminating substances. The method employs a magnetically responsive particle which is surface-coated with an oligonucleotide-binding partner. The coated particle is added to a biological sample. Oligonucleotides bind to the particle by virtue of their interaction with the binding partner and are then separated from the sample by application of a magnetic field.

Accordingly, it is an object of the invention to provide a method of purifying a target oligonucleotide analyte by providing a sample containing the target analyte, contacting the sample with a magnetically responsive particle to which an oligonucleotide analyte-binding partner has been linked, incubating the sample with the particle, thereby forming a particle-binding partner-analyte complex, exposing the sample to a magnetic field, and separating the complex from the sample.

DETAILED DESCRIPTION OF THE INVENTION

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA technology, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. *See, e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Immobilized Cells and Enzymes* (IRL press, 1986); the series, *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); and *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., 1986, Blackwell Scientific Publications).

A. Definitions:

Before the invention is described in detail, it is to be understood that this invention is not limited to specific analyte-binding partners, magnetically responsive particles or coating techniques as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an analyte" includes mixtures of analytes, reference to "an analyte-binding partner" includes mixtures of two or more such binding partners, and the like. In this regard, it is important to note that the techniques of the present invention may be used to purify multiple analytes from a sample, e.g., as captured on a magnetically responsive particle.

In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

As used herein, the term "oligonucleotide" include double- and single-stranded DNA, as well as double- and single-stranded RNA. The term "oligonucleotide analyte" refers to a single- or double-stranded oligonucleotide to which a target analyte-binding partner will bind. An analyte oligonucleotide may be from a variety of sources, e.g., human or other mammalian biological fluids or tissues, including blood, urine, cerebrospinal fluid, stool, sputum, or wound exudates, genetic DNA or RNA from bacteria or viruses, food stuffs, environmental materials, etc., and may be prepared for the purification procedure by a variety of means, e.g., proteinase K/SDS, chaotropic salts, or the like. The term "oligonucleotide analyte" is used interchangeably herein with the terms "analyte" and "target analyte."

The term "analyte" as used herein is intended to mean a molecular species to be purified. As noted above, analytes are purified by virtue of their binding to a magnetically responsive particle which has been surface-coated with an analyte-binding partner, to create a particle-binding partner-analyte complex. The complex is thereafter separated from the sample by applying a magnetic field to the sample, thereby immobilizing the analyte against further processing to remove the remaining components of the sample. The term "analyte" is also intended to encompass molecular species which are functionally equivalent to analytes of interest in a particular context. Thus, the term "analyte" includes analyte analogs, analyte fragments, and the like.

The terms "analyte-binding partner," "target-binding partner" and "oligonucleotide-binding partner" as used herein are intended to encompass molecules which will bind to analyte oligonucleotides. Analyte-binding partners may be proteins which recognize general structural features of the target analyte, i.e., they may bind to single-stranded or double-stranded oligonucleotides, or they may recognize specific nucleotide sequences in the target analyte.

The terms "isolation" and "purification" as used herein are intended to mean that a composition containing a specific oligonucleotide analyte, in addition to other materials including other oligonucleotide species, may be significantly enriched in that oligonucleotide, i.e., such that the composition then comprises the oligonucleotide in substantially pure form. Optical or fluores-

The method disclosed herein combines rapid lysis of cellular components in the sample, binding of the released target oligonucleotide to specific oligonucleotide-binding partners linked to magnetically responsive particles and separation of the oligonucleotide from the crude solution by application of a magnetic field.

The use of magnetically responsive particles to effect separation of components from biological samples has been reviewed (Hirschbein et al. (1982) *Chemtech* March 1982:172-179; Pourfarzaneh (1982) *The Ligand Quarterly* 5:41-47; and Halling et al. (1980) *Enzyme Microb. Technol.* 2:2-10).

In order to purify oligonucleotides, e.g., DNA, out of a sample which is complex and mixed such as a blood sample, the cellular components in the sample are first subjected to lysing agents such as lysozyme, SDS, Triton X-100, or the like. Agents and methods for lysing cells are well known in the art. *See, for example*, Birnboim (1992) *Meth. Enzymol* 216:154-160 and Sambrook et al., *supra*.

For example, oligonucleotides can be released from biological systems via a variety of methods including the chemical action of detergents, bases, acids, chaotropes, organics and mixtures of these chemicals on samples. Physical methods of processing samples can be employed and include pressure, heat, freeze-thaw cycles and sonication with or without glass beads. Further, combinations of physical and chemical methods have can be used to prepare samples such as chemical lysis followed by sonication.

Magnetically responsive particles which are surface-coated with oligonuleotide-binding partners are added to a reaction vessel, e.g., a borosilicate glass, polypropylene or polycarbonate test tube, holding a sample containing or suspected of containing a target oligonucleotide analyte to form a suspension. The suspension is incubated for a sufficient duration to allow the surface-coated particles to bind to the desired target, thereby forming a particle-binding partner-analyte complex. The reaction vessel is then placed in close proximity to a source of magnet field to immobilize the complex to the inner surface of the reaction vessel. After decanting, aspirating or otherwise removing the supernatant fluid from the vessel, the particle-binding partner-analyte complex is washed by adding an appropriate buffer or other reagent, removing the vessel from the magnet and resuspending the particle-target complex. The wash procedure may be repeated as often as desired.

Magnetically responsive particles are well known in the art (*see* U.S. Patent No. 4,672,040 to Josephson) and are commercially available from, for example, Dynal®, Inc. (Lake Success, NY) and Bangs Laboratories, Inc. (Carmel, IN). Magnetically responsive latex particles may be made of divinylbenzene, polystyrene, or other polymers, copolymers, and terpolymers, and have -COOH or $-NH_2$ surface groups, or other defined chemical functionalities such as aldehyde, aliphatic amine, amide, aromatic amine, haloalkyl, hydrazide or hydroxyl by which biomolecules may be covalently, ionically, adsorptively or otherwise bound. The particles are magnetic, paramagnetic or otherwise responsive to an applied magnetic field. The preferred size of the magnetically responsive particle ranges from 0.5 to 5.0 μ. The particles preferably have an iron oxide content of approximately 10 to 60% by weight and a surface -COOH content of between about 20 to 200 μ equivalents per gram of particles.

The source of the magnetic field may be a permanent magnet, e.g., a ferro- or ferrimagnetic material, or an induced magnet, i.e., an electromagnet. One such magnet is a neodynium-iron-boron permanent magnet (Dynal®, Inc.).

Oligonucleotide analyte-binding partners may be prepared by procedures which have been described in the literature. The binding partners may be purified from biological samples, prepared synthetically from known amino acid sequences, prepared from motifs which have been shown to bind oligonucleotides, e.g., leucine-zipper proteins (Vinson et al. (1989) *Science* 246:911-916) or Cys_2His_2 zinc-finger proteins (Desjarlais et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:2256-2260), prepared recombinantly or obtained commercially, e.g., *E. coli* rec A protein (Sigma). In addition, oligonucleotide-binding partners may be isolated using magnetically responsive particles surface-coated with oligonucleotides, or fragments thereof. *See*, Gabrielsen et al. (1993) *Meth. Enzymol.* 218:508-525.

The surface of the magnetically responsive particles may be prepared for linking analyte-binding partners thereto by any method well known in the art. One preferred method involves functionalizing the surface prior to coating with a binding partner.

The surface of the magnetically responsive particles is treated with an organosilane coupling agent to functionalize the surface. The organosilane coupling agent is preferably represented by the formula $R_nSiY_{(4-n)}$ where: Y represents a hydrolyzable group, e.g., alkoxy, typically lower alkoxy, acyloxy, lower acyloxy, amine, halogen, typically chlorine, or the like; R represents a nonhydrolyzable organic radical that possesses a functionality which enables the coupling agent to bond with organic resins and polymers; and n is 1, 2 or 3. One example of such an organosilane coupling agent is 3-glycidoxypropyltrimethoxysilane ("GOPS"), the coupling chemistry of which is well-known in the art. *See*, for example, Arkins, "Silane Coupling Agent Chemistry," *Petrarch Systems Register and Review*, Eds. Anderson et al. (1987). Other examples of organosilane coupling agent are (γ-aminopropyl)triethoxysilane and (γ-aminopropyl)trimethoxysilane. Still other suitable coupling agents are well known to those skilled in the art. In the next step, the organosilane coupling agent, now covalently bound to the substrate surface, is derivatized, if necessary, to provide for surface reactive groups which will bind the binding-partner coating. For example, if the organosilane coupling agent provides for surface vicinal diol groups, these can be converted to reactive aldehyde groups by conventional methods (e.g., by reaction

with 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 5.5. The particles are then recovered by application of a magnetic field and the supernatant is discarded. The particles are resuspended in 0.1 M diethanolamine buffer, pH 10.5, containing 45 mg/ml water soluble carbodiimide, e.g., 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide, stirred at room temperature for 30 minutes and washed three times with fresh diethanolamine buffer.

The washed particles are resuspended in diethanolamine buffer with 2-7 mg/ml SSB or RecA protein, stirred for 60 minutes at room temperature and washed with buffer. The derivatized particles are stored at 4°C alter saturating them with 1 mg/ml bovine serum albumin.

B. Purification of Gonococcal DNA-binding proteins.

Gonococcocal whole-cell DNA is purified from cultured *N. gonorrhoeae* 31426 (American Type Culture Collection, Rockville, MD) as described by Dorward et al., *supra*. Ethanol-precipitated DNA is washed once with 70% ethanol and then dissolved at 1 mg/ml in diethanolamine buffer.

SSB or RecA protein-derivatized magnetically responsive particles (~ 5 mg) are added to the DNA solution. The suspension is allowed to incubate for 10-20 minutes and then exposed to a magnetic field by placing a magnet (neodynium-iron-boron permanent magnet, Dynal®, Inc.); the beads are rapidly collected in a firm pellet. After the supernatant is aspirated, the magnet is removed and the pellet is washed with buffer 3 times. The final wash is done with buffer containing 1.0 M NaCl to dissociate the bound DNA from the derivatized beads. The purified DNA is analyzed by agarose gel electrophoresis as described in Sambrook et al., *supra*.

Claims

1. A method for purifying a target oligonucleotide analyte comprising:

   (a) providing a sample containing or suspected of containing the target;
   (b) contacting the sample with an oligonucleotide analyte-binding partner, wherein the binding-partner is linked to a magnetically responsive particle;
   (c) incubating the sample with the particle, thereby forming a particle-binding partner-analyte complex;
   (d) exposing the complex to a magnetic field;
   (e) separating the complex from the sample; and
   (f) releasing the target analyte from the complex.

2. The method of claim 1, wherein the target oligonucleotide analyte is RNA or DNA.

3. The method of claim 2, wherein the target oligonucleotide is DNA.

4. The method of claim 1, wherein the analyte-binding partner is a protein or an antibody.

5. The method of claim 4, wherein the analyte-binding partner is a protein.

6. The method of claim 5, wherein the protein is a DNA-binding protein.

7. The method of claim 6, wherein the DNA-binding protein is selected from the group consisting of leucine-zipper proteins, Cys_2His_2 zinc-finger proteins, *E. coli* rec A protein, the IE-2 protein of human cytomegalovirus, the *Neisseria gonorrhoeae* DNA-binding protein, filamentous phage M13 gene V product, filamentous phages fd gene V product, phage Ike PIKE protein, bacteriophage T4 gene 32 protein, bacteriophage T7 gene 2.5 protein, *E. coli* F sex factor-coded SSF protein, SSB protein, N4 SSB, herpes simplex virus type 1 major DNA binding protein ICP8, herpes simplex virus type 2 major DNA binding protein ICP8, HSV DNA binding protein ICP4, HSV DNA binding protein ICSP 11/12, *S. cerevisiae* centromere DNA element I binding protein, UV-inducible, damage-specific DNA binding protein, MeCP2, SAF-A, DNA polymerase, *lac* repressor, *lac* operator, adenovirus major late transcription factor, and Drosophila heat-shock activator protein.

8. The method of claim 2, wherein the target oligonucleotide is RNA.

9. The method of claim 5, wherein the protein is an RNA-binding protein.

10. The method of claim 9, wherein the RNA-binding protein is selected from the group consisting of eukaryotic initiation factor-3, calreticulin, SecA protein, AUF1, RNA-binding protein K, RNA-binding protein H16, Tat, and nucleolin.

11. The method of claim 1, wherein the binding partner is linked to the magnetically responsive particle covalently, ionically or adsorptively.

12. The method of claim 11, wherein the binding partner is linked to the magnetically responsive particle covalently.

13. The method of claim 1, wherein the magnetic field is generated by a permanent or an induced magnet.